Human HRD1 protects against ER stress-induced apoptosis through ER-associated degradation[1]

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Abstract **Stresses that impair the function of the endoplasmic reticulum (ER) lead to an accumulation of unfolded protein in the ER. Under these conditions, the expression of a variety of genes involved in preventing the accumulation of the unfolded proteins is induced. Yeast Hrd1p is an ER stress-inducible ER membrane protein that acts as a ubiquitin ligase (E3) with a RING finger motif and plays a role in the ubiquitination of proteins in the ER. We report here the identification and characterization of a human homolog to yeast Hrd1p. The predicted structures are highly conserved from yeast to humans. Indeed, human HRD1 was localized to the ER and ubiquitinated its substrates. Furthermore, it was found that human HRD1 was up-regulated by ER stress via IRE1 and ATF6, which are ER stress transducers. Interestingly, 293 cells stably expressing wild-type HRD1, but not the C329S mutant, afforded resistance to ER stress-induced apoptosis. These results suggest that the production of HRD1 is up-regulated to protect against ER stress-induced apoptosis by degrading unfolded proteins accumulated in the ER.**

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Key words: Endoplasmic reticulum; Endoplasmic reticulum-associated degradation; Hrd1p; Ubiquitin ligase; Unfolded protein response; IRE1

1. Introduction

The exposure of cells to various forms of stress interfering with the functions of the endoplasmic reticulum (ER) leads to the accumulation of unfolded protein in the ER lumen. Under these conditions, known as ER stress, a signal transduction pathway, called the unfolded protein response (UPR), is activated to increase the expression of ER stress response genes, such as chaperone protein genes [1].

The UPR has been well defined in *Saccharomyces cerevisiae* [2]. When unfolded proteins accumulate in the ER lumen, Ire1p, which is a protein kinase and ribonuclease in the ER membrane, senses them and activates itself by *trans*-autophosphorylation, and eventually induces the gene expression. A recent microarray analysis in yeast has shown that the expression of a variety of genes is induced via Ire1p in response to ER stress [3]. This result revealed that UPR-regulated genes consist of another group involved in ER-associated degradation (ERAD), including *HRD1*/*DER3*, *HRD3*, *DER1* and *UBC7*. The proteins encoded by these genes serve to remove unfolded proteins by retrograde transport from the ER back to the cytosol and subsequent degradation using the ubiquitin-proteasome system [4–6]. Hrd1p is a ubiquitin ligase (E3) in the ER membrane with its N-terminal hydrophobic region in multiple transmembrane spans, and with a C-terminal hydrophilic region containing a RING-H2 motif required for binding to ubiquitin-conjugating enzyme (E2). Hrd1p participates in the degradation of Hmg2p, a homolog of mammalian hydroxymethylglutaryl-coenzyme A reductase [7–9].

Two Ire1p homologs (α and β) exist in mammalian cells and serve as activators of the UPR [10,11]. In addition, ATF6 has been characterized as another UPR inducer, which is processed and liberated from the ER membrane in response to ER stress, and subsequently translocates from the ER membrane to the nucleus as a transcription factor [12,13]. Although many more genes related to the UPR should exist in mammals than yeast, little is known as yet, especially of the mammalian ERAD system.

Interestingly, it was recently suggested that neuronal cells in neurodegenerative diseases undergo apoptosis induced by ER stress, due to the accumulation of unfolded proteins in the ER with the collapse of the UPR system [14–17]. Therefore, it is necessary to identify novel mammalian molecules induced by the UPR and elucidate the contribution to the prevention of ER stress-induced apoptosis. We report here that a human homolog of Hrd1p was identified and characterized as a ubiquitin ligase, and that its expression was induced by the UPR to protect cells from ER stress through ERAD.

2. Materials and methods

2.1. Cloning and expression vector construction

Human HRD1 was cloned from HEK293 cDNA using KIAA1810-specific primers. Amplified HRD1 cDNA was ligated into the vector pcDNA6/Myc-His (Invitrogen), expressing Myc (c-Myc) and polyhistidine (6×His) epitopes at the C-terminus of the inserted sequence. IRE1 and ATF6 (a truncated form encoding the cytoplasm region corresponding to amino acids 1–373) were amplified with a hemag-

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[1] The nucleotide sequence data reported are available in the DDBJ/EMBL/GenBank databases under the accession number AB085847.

Abbreviations: ER, endoplasmic reticulum; ERAD, ER-associated degradation; HRD, hydroxymethylglutaryl reductase degradation; UPR, unfolded protein response; E3, ubiquitin-protein ligase; E2, ubiquitin-conjugating enzyme; RT-PCR, reverse transcription-polymerase chain reaction; UBL, ubiquitin-like; SEL, suppressor/enhancer of lin-12; PS, presenilin; FAD, familial Alzheimer's disease

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glutinin epitope at the C-terminus or N-terminus, respectively, and then cloned into the mammalian expression vector pCR3.1 (Invitrogen). The HRD1-C329S and IRE1-K599A mutants were constructed by PCR using the overlapping method.

2.2. Cell culture

Human embryonic kidney (HEK) 293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat-inactivated fetal calf serum, 100 U/ml of penicillin, and 100 μg/

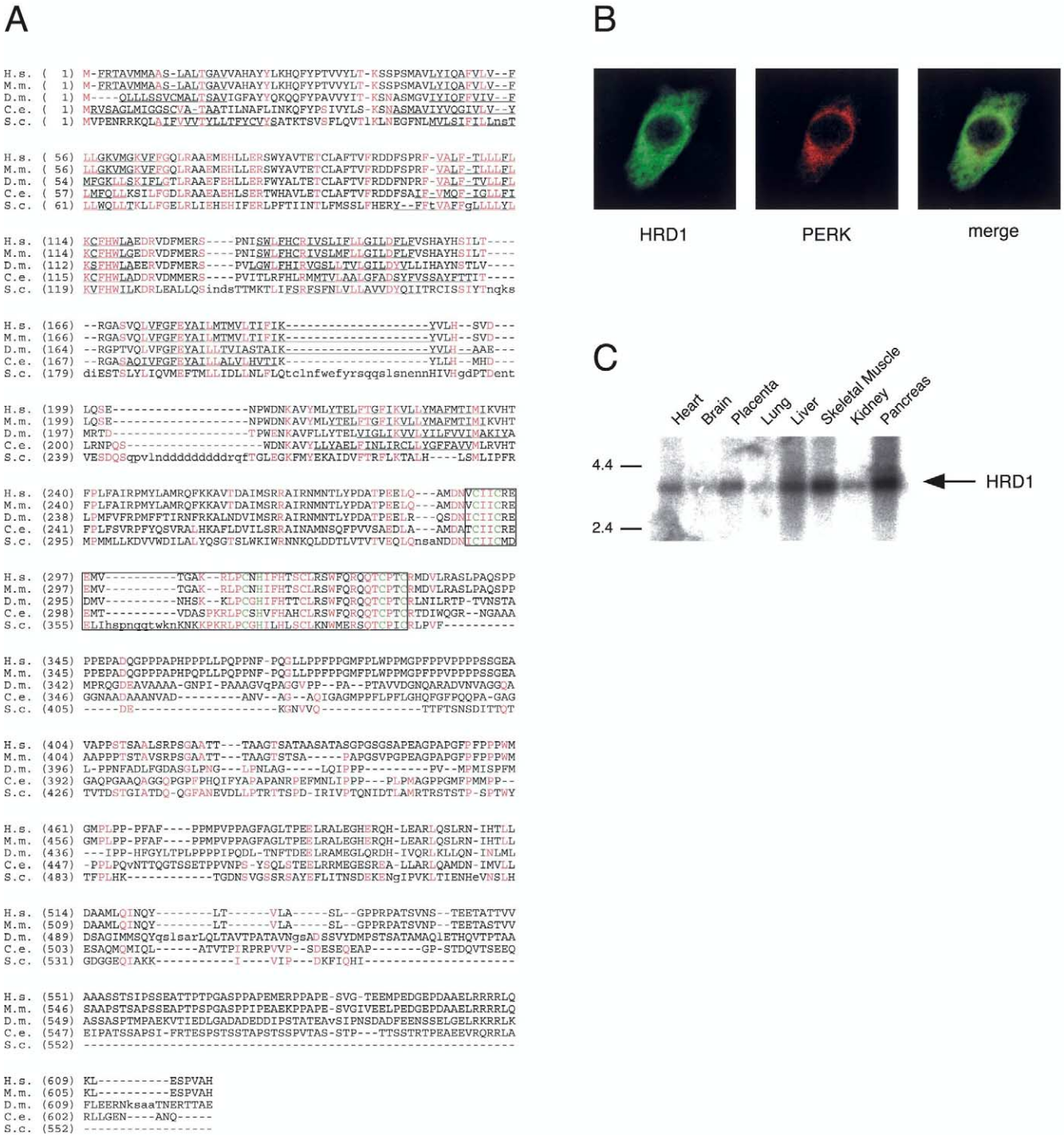

Fig. 1. Amino acid sequence, subcellular localization and tissue distribution of human HRD1. A: Amino acid sequence alignment of Hrd1p homologs. H.s. (*Homo sapiens*) is human HRD1. M.m. (*Mus musculus*), D.m. (*Drosophila melanogaster*) and C.e. (*Caenorhabditis elegans*) are putative proteins, which are similar to yeast Hrd1p (S.c.: *S. cerevisiae*) in the databases. Identical sequences with Hrd1p are shown in red. Predicted transmembrane regions are underlined. The RING finger domain is boxed, and its conserved motif is green. An asterisk indicates the site of a point mutation. B: Subcellular localization of HRD1. HRD1 tagged with a Myc epitope at its C-terminus was transiently overexpressed in CCF-STTG1 cells. Myc-HRD1 and endogenously expressed PERK were detected by immunofluorescence microscopy using anti-Myc (left panel) and anti-PERK (center panel) antibodies, respectively. The right panel is the merged image of the left and center panels. C: Tissue distribution of HRD1 in humans. A human multiple tissue Northern™ blot (2 μg of poly(A)$^+$ RNA/lane, Clontech) was hybridized with a ^{32}P-labeled HRD1 cDNA probe. Positions of standard RNA markers are shown on the left with their size.

ml of streptomycin at 37°C in a humidified 5% CO_2/95% air atmosphere. Human astrocytoma CCF-STTG1 cells were maintained in RPMI medium. 293 cell lines stably expressing HRD1s were generated by transfection with wild-type (wt) or mutated (C329S) HRD1-pcDNA6, and then selected in medium containing 5 μg/ml of blasticidin (Invitrogen).

2.3. Immunohistochemistry

To detect the expression of HRD1 at the cell level, CCF-STTG1 cells were transfected with the expression vector wt HRD1 using the LipofectAMINE 2000 reagent, fixed with 1% paraformaldehyde phosphate buffer for 5 min, and permeabilized with 0.5% Triton X-100 for 5 min. The cells were then stained for the presence of proteins with ant-Myc (9E10, Oncogene) and anti-PERK (Santa Cruz) antibodies.

2.4. Immunoprecipitation and immunoblotting

293 cells were transfected with wt or C329S HRD1 expression vectors and treated with 5 μM MG132 (Peptide Institute, Japan) for various periods before being harvested. The cells were lysed in lysis buffer (10 mM Tris–HCl [pH 7.4], 150 mM NaCl, 5 mM EDTA, 10% glycerol and 0.5% Triton X-100) with complete protease inhibitors (Roche Diagnostics, Germany) at 4°C for 20 min, and then lysates were centrifuged. The supernatant was immunoprecipitated using anti-Myc antibody and protein G-Sepharose (Amersham Pharmacia Biotech, UK). The whole cell lysates were subjected to Western blot analysis using anti-multiubiquitin (FK2, Nippon Bio Test Laboratory, Japan) or anti-Myc antibodies. Subsequently, the bands were detected using Ig-horseradish peroxidase-conjugated antibody and the enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech, UK).

2.5. Cell death assay

The apoptotic cells were detected with cytoplasmic histone-associated DNA fragments generated by endonuclease cleavage using the Cell Death Detection ELISA (Roche Diagnostics, Germany) according to the manufacturer's protocol. The results are expressed as the fold increase in optical density, resulting from the activity of peroxidase-conjugated anti-DNA antibody complexed with cytoplasmic nucleosomes of treated cells, compared with the control.

3. Results

3.1. Identification of human HRD1

To identify the human homolog of yeast Hrd1p, we searched the protein databases (GenBank CDS translations, PDB, SwissProt, PIR and PRF) with NCBI BLAST using the yeast Hrd1p amino acid sequence. An already isolated human clone, KIAA1810 (GenBank accession number AB058713), the function of which is unknown, was discovered as a candidate for the human homolog of Hrd1p. We decided to study this clone, termed HRD1. The human HRD1 protein has 26.2% overall identity to yeast Hrd1p in amino acid sequence, the RING finger domain showing 45.1% identity. Moreover, we searched for other Hrd1p homologs in a number of species, and found that the N-terminal half of the protein is comparatively well conserved in the predicted six transmembrane domains and the RING finger domain between species compared to the C-terminal side (Fig. 1A). Next, to examine the subcellular localization of human HRD1 protein, Myc-tagged HRD1 was overexpressed in CCF-STTG1 cells. The HRD1 protein co-localized with endogenous PERK, an ER stress sensor protein in the ER membrane (Fig. 1B), indicating that localization of human HRD1 is conserved between yeast and humans. Moreover, the distribution of HRD1 in human organs was examined by Northern blot analysis using a human HRD1-specific probe. Human HRD1 was ubiquitously expressed in all organs and detected as a single species of 4.2 kb. In particular, the HRD1 mRNA was abundant in pancreas, liver and skeletal muscle (Fig. 1C).

3.2. HRD1 has ubiquitin ligase activity

Yeast Hrd1p acts as a ubiquitin ligase in the ERAD system. To elucidate whether the function of HRD1 is conserved in humans, we investigated the E3 activity of human HRD1. We performed an immunoprecipitation assay using Myc-HRD1 overexpressed in 293 cells, followed by Western blotting with anti-multiubiquitin monoclonal antibody. The co-immunoprecipitates with Myc-HRD1 were detected as slowly migrating bands by an anti-multiubiquitin antibody, and the intensity of this band was strengthened on the addition of MG-132, a proteasome inhibitor, in a time-dependent manner (Fig. 2, upper panel). These high molecular weight smear bands seem to involve unidentified substrates ubiquitinated

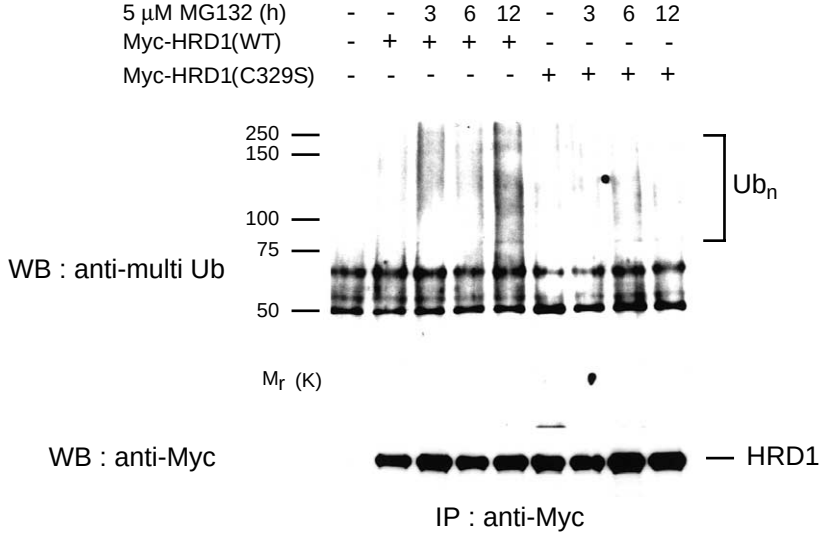

Fig. 2. HRD1 has an E3 ubiquitin ligase activity. 293 cells were transfected with empty vector plasmid, Myc-tagged wt or C329S HRD1 cDNA, and treated with 5 μM MG-132 for the periods indicated. At 48 h after transfection, whole cell lysate was extracted, immunoprecipitated with anti-Myc monoclonal antibody (9E10), and Western blotted with anti-multiubiquitin monoclonal antibody (FK2) (upper panel) or anti-Myc monoclonal antibody (lower panel).

and co-immunoprecipitated by HRD1, since the form of auto-ubiquitinated HRD1 detected by the anti-Myc antibody was different from that of substrates detected by the anti-multi-ubiquitin antibody (data not shown). In yeast Hrd1p, a cysteine at 399 in the RING finger domain is necessary for the E3 activity, since the mutated Hrd1p C399S fails to bind the E2 proteins and ubiquitinate its substrates [9,18]. A human HRD1 mutant defective in E3 activity was constructed in which the conserved Cys-329 corresponding to yeast Cys-399 was replaced with serine, on the basis of the complete conservation of the consensus sequence in the RING finger motif of HRD1 (Fig. 1A). C329S HRD1 was transfected into 293 cells, and the cells subsequently immunoprecipitated with an anti-Myc antibody. As expected, multiubiquitinated proteins were not observed in mutated HRD1-expressing cells (Fig. 2, upper panel). These results indicate that human HRD1 has E3 activity and can ubiquitinate its substrates.

3.3. *HRD1 expression is induced by ER stress*

In yeast, the expression of a number of genes related to ERAD is up-regulated by the UPR [2]. Next, to elucidate whether the induction mechanism for HRD1 is conserved in mammals, we examined the expression of mRNA under ER stress using 293 cells. Cells were treated with an ER stress-inducing reagent, such as thapsigargin (sarco/endoplasmic reticulum Ca^{2+} ATPase inhibitor), tunicamycin (*N*-glycosylation inhibitor), brefeldin A (ER–Golgi transport inhibitor) or dithiothreitol (DTT; reducing agent), and mRNA levels of HRD1 were then measured by reverse transcription-polymerase chain reaction (RT-PCR). A marked increase was observed in the HRD1 mRNA levels in response to these ER stresses (Fig. 3A,B), although other forms of stress, including heat shock, produced little change (Fig. 3B). Moreover, CHOP and Parkin, known as ER stress response gene products [19,20], were concomitantly produced in response to these reagents (Fig. 3A). In contrast, HSP70, a cytosolic stress response gene product, exhibited no increase at the mRNA level (Fig. 3B). In yeast, Ire1p mediates the expression of *HRD1* and other ER stress-induced genes in response to ER stress [2]. On the other hand, in mammals, in addition to IRE1s (α and β), ATF6 participates in the induction of ER stress response genes [21–23]. We next investigated whether overexpression of IRE1 and ATF6 can up-regulate the production of human HRD1, as that of Ire1p induces an increase in yeast *HRD1*. Overexpression of IRE1α and ATF6 (1–373; cytoplasmic domain worked as a transcription factor) produced an increase in mRNA of human HRD1 (Fig. 3C). Furthermore, we examined the dominant negative effect of the IRE1-K599A mutant, in which the ATP binding site at lysine residue 599, required for the kinase activity, was replaced with alanine, on the expression of HRD1 induced by ER stress. The level of HRD1 mRNA expression induced by tunicamycin was attenuated in 293 cells stably expressing the IRE1-K599A mutant, compared with normal 293 cells (Fig. 3D). On the other hand, the ER stress-induced CHOP expression was also partially reduced by expression of IRE1-K599A (Fig. 3D), indicating that this mutant could effectively inhibit the UPR pathway. These results indicate that the mechanism by which UPR induces expression of HRD1 is conserved from yeast to mammals in the pathway via IRE1. In addition, ATF6 is also capable of inducing HRD1 expression.

3.4. *HRD1 suppresses ER stress-induced apoptosis*

It has been reported that Parkin, an E3 ubiquitin ligase involved in ERAD, is up-regulated by the UPR and protects cells from ER stress-induced apoptosis by accelerating the

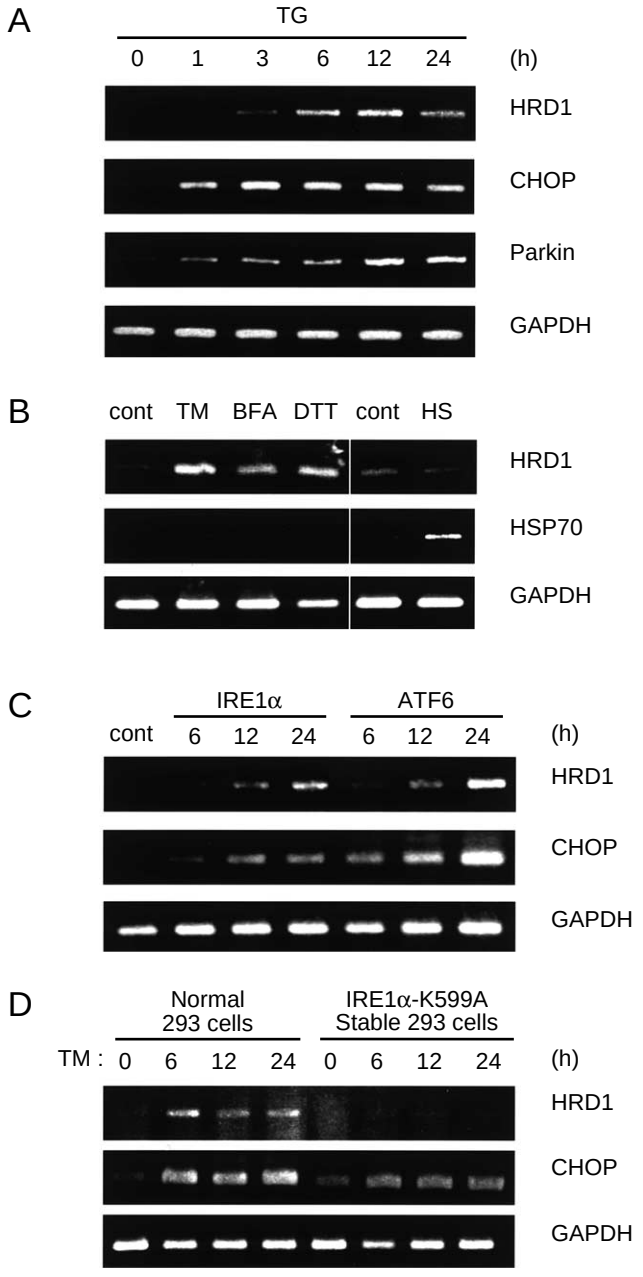

Fig. 3. HRD1 is up-regulated by the unfolded protein response. A: Time-dependent induction by ER stress in HRD and other ER stress response genes. 293 cells were treated with thapsigargin (TG; 1 μM) for the periods indicated, then total RNA was prepared and subjected to RT-PCR using specific primers (HRD1, CHOP, Parkin, GAPDH). B: The induction of HRD1 by various forms of stress. 293 cells were treated with tunicamycin (TM; 2 μg/ml), brefeldin A (BFA; 1 μg/ml), DTT (2 mM) or heat shock (HS; 42°C, 1 h) for 6 h. C: Effect of overexpressing IRE1 and ATF6 on the expression of HRD1. 293 cells were transfected with IRE1 or ATF6 (N-terminal side in the cytosol acting as a transcription factor) using LipofectAMINE 2000. D: Effect of dominant negative IRE1-K599A mutant on ER stress-induced HRD1 expression. Normal 293 cells and those stably expressing IRE1-K599A were treated with 2 μg/ml of tunicamycin for the periods indicated.

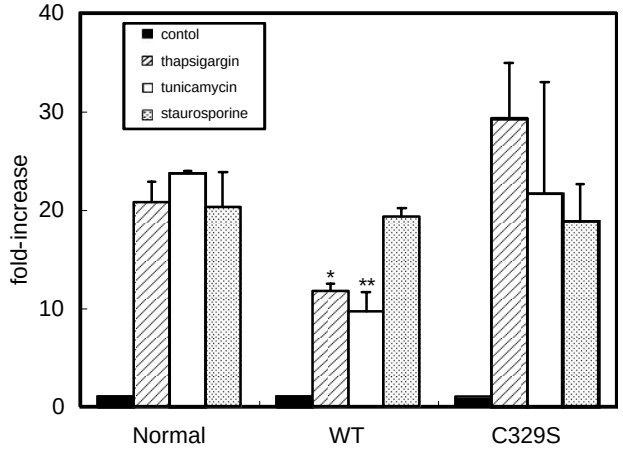

Fig. 4. HRD1 rescues the cells from ER stress-induced apoptosis. Normal, wild-type (WT) or mutant (C329S) HRD1-expressing 293 cells were treated with thapsigargin (1 μM), tunicamycin (10 μg/ml), and staurosporine (0.5 μM). Forty-eight hours after incubation, the apoptotic cells were detected by ELISA of cytoplasmic nucleosomes, as described in Section 2. Values represent the means ± S.E.M. of three independent experiments. Asterisks represent significantly different values of wt HRD1-expressing cells from those of normal 293 cells (*$P < 0.05$; **$P < 0.01$; Student's t-test).

degradation of unfolded proteins accumulated in the ER lumen [16,20]. Therefore, up-regulation of HRD1 expression might rescue cells from ER stress-induced apoptosis, if HRD1 serves as an E3 to degrade the unfolded protein in the ER. To investigate whether HRD1 suppresses ER stress-induced apoptosis, we performed a apoptosis assay using 293 cells stably expressing human HRD1s. Wt HRD1-expressing cells were significantly more resistant to ER stress, including thapsigargin and tunicamycin, than normal 293 cells. In contrast, the C329S HRD1 mutant-expressing cells, lacking E3 activity, showed no resistance to ER stress-induced apoptosis compared with normal 293 cells (Fig. 4). On the other hand, no difference among wt, mutant HRD1-transfected and untransfected cells was observed in the apoptosis induced by staurosporine (Fig. 4B). These results indicate that the protective effect of HRD1 against apoptosis is dependent on its E3 activity and specific to ER stress.

4. Discussion

We have identified a human homolog of Hrd1p by homology search and using prediction programs for localization and domains, and demonstrated that human HRD1 has functional homology to yeast Hrd1p. Moreover, we have shown that its expression is induced by ER stress, resulting in protection against ER stress-induced apoptosis through the ubiquitin-proteasome system.

There is further evidence that human HRD1 is a bona fide homolog of yeast Hrd1p. Hrd3p, another UPR-inducible ERAD protein, interacts with Hrd1p and mediates the regulation of Hrd1p stability and activity [7]. HRD1 can interact with suppressor/enhancer of lin-12 (SEL1L), which is a candidate homolog of Hrd3p (data not shown). Although SEL1L has been cloned as a human homolog of *Caenorhabditis elegans* sel-1, little is known about its function in relation to ERAD [24]. We have identified SEL1L by the same strategy, and found that it too is induced by the UPR (data not shown). Therefore, these results give us confidence that HRD1 is a homolog of Hrd1p.

Parkin, another cytosolic E3 protein involved in mammalian ERAD, has two RING finger domains and a ubiquitin-like (UBL) domain that interacts with 26S proteasome. However, HRD1 has only a RING finger domain, no UBL domain, indicating that HRD1 may require the cooperation of another UBL protein for proteasomal degradation. On the other hand, Kokame et al. and we have identified ER stress-inducible proteins, Herp and ubiquilin, respectively [25,26]. Both have a UBL domain and exist in the ER as transmembrane protein. These reports suggest that HRD1 and UBL proteins, including Herp and ubiquilin, could cooperate in ubiquitination and interaction between the substrates and 26S proteasome.

Induction of human HRD1 expression is mediated by an IRE1α-dependent pathway in response to ER stress. This is consistent with results that the expression of yeast Hrd1p and other ERAD-related genes is up-regulated by Ire1p [2]. This suggests that the mechanism by which UPR induces expression of HRD1 is conserved from yeast to mammals. On the other hand, the involvement of an ATF6-mediated pathway(s) in the expression of ERAD genes, including HRD1, remains unclear, since no ATF6 counterpart exists in yeast. Although ER stress response genes have ATF6 binding elements, ERSE, in the promoters, no ERSE motif exists in the putative HRD1 promoter [14]. Further study is necessary to elucidate the mechanism by which ATF6 induces the expression of mammalian genes, particularly in ERAD.

Northern blot analysis revealed that HRD1 is expressed at a high level in pancreas. Moreover, expression of SEL1L is also abundant in the tissue [24]. It was reported that IRE1α is expressed predominantly in pancreas [10]. These results suggest that the ERAD system is essential for the function of ER-developed tissues, including the pancreas, and that IRE1α plays a key role in the regulation of ERAD-related gene expression in those tissues.

HRD1 has a protective effect dependent on the E3 activity against ER stress. This finding suggests that ERAD proteins induced by ER stress play a critical role in preventing ER stress-induced apoptosis. In fact, overexpression of Parkin can suppress apoptosis induced by ER stress through the E3 activity [20]. In this study, we also demonstrated the protective effect of overexpression of HRD1 against ER stress-induced apoptosis. These results suggest that HRD1, Parkin and other unidentified ubiquitin ligases involved in ERAD are concomitantly up-regulated in response to ER stress via the UPR signaling pathways, resulting in suppression of apoptosis through degradation of unfolded proteins by those components.

Furthermore, the ERAD action seems to have a great influence on the accumulation of proteins in neurons. For example, the mutations in the presenilin-1 (PS1) gene that cause early onset familial Alzheimer's disease (FAD) attenuate the UPR by disturbing the activation of IRE1 and ATF6, increasing the vulnerability to ER stress by altering the expression of the ER chaperone [14,15]. These reports and our findings suggested that mutations in PS1 attenuate the expression of IRE1 and ATF6-dependent genes related to ERAD, including HRD1 and SEL1L, resulting in an accumulation of unfolded proteins in the ER, and cause FAD. Therefore, studying the induction mechanism of ERAD genes and protective effect

against ER stress in mammals may provide a clue to the mechanism behind neurodegenerative diseases.

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